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In polarized epithelial cells, components of the membrane fusion machinery, the t-SNAREs syntaxin 2, 3, 4 and SNAP-23 are differentially localized at the apical and/or basolateral plasma membrane domains. Surprisingly, all of these t-SNAREs redistribute to intracellular locations when cells lose their cellular polarity during mammary carcinogenesis. Apical SNAREs re-localize to the previously characterized vacuolar apical compartment (VAC) while basolateral SNAREs redistribute to a novel organelle that appears to be the basolateral equivalent of the VAC. Both 'intracellular plasma membrane compartments' are associated with the actin cytoskeleton and receive membrane traffic from cognate apical or basolateral pathways, respectively. These findings demonstrate a fundamental shift in plasma membrane traffic towards intracellular compartments while protein sorting is preserved when epithelial cells lose their cell polarity.

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TABLE OF CONTENTS

	PAGE
FRONT COVER	1
SF 298, REPORT DOCUMENTATION PAGE	2
FOREWORD	3
TABLE OF CONTENTS	4
INTRODUCTION	5
BODY	6-11
KEY RESEARCH ACCOMPLISHMENTS	11
REPORTABLE OUTCOMES	11
CONCLUSIONS	11
REFERENCES	12-15
APPENDICES	15

5. Introduction

In polarized epithelial cells, components of the membrane fusion machinery, the t-SNAREs syntaxin 2, 3, 4 and SNAP-23 are differentially localized at the apical and/or basolateral plasma membrane domains. Surprisingly, all of these t-SNAREs redistribute to intracellular locations when cells lose their cellular polarity during mammary carcinogenesis. Apical SNAREs re-localize to the previously characterized vacuolar apical compartment (VAC) while basolateral SNAREs redistribute to a novel organelle that appears to be the basolateral equivalent of the VAC. Both 'intracellular plasma membrane compartments' are associated with the actin cytoskeleton and receive membrane traffic from cognate apical or basolateral pathways, respectively. These findings demonstrate a fundamental shift in plasma membrane traffic towards intracellular compartments while protein sorting is preserved when epithelial cells lose their cell polarity.

6. Body

Traffic between membranous compartments is mediated by the SNARE machinery in virtually all membrane traffic pathways investigated so far (14, 15, 27, 33). During vesicle docking, membrane proteins on the vesicle (v-SNAREs) and the target membrane (t-SNAREs) bind to each other to form a complex which ultimately leads to fusion of the lipid bilayers. One aspect of the SNARE hypothesis is that successful membrane fusion requires the binding of matching combinations of v- and t-SNAREs thereby ensuring the necessary specificity of vesicle fusion. Accordingly, each membrane organelle and each class of transport vesicles should be defined by a certain set of t- and v-SNARE isoforms. Many SNAREs have been identified to date and protein sequence analysis has shown that v- and t-SNAREs of the currently known SNARE sub-families are evolutionarily related to each other and belong to a common superfamily (41, 43). It is conceivable that the specificity of vesicle fusion is not directly determined by t-SNARE/v-SNARE interactions *per se* but rather by interactions involving larger complexes including SNAREs and their regulatory proteins such as those of the rab and sec1 protein families (4, 12, 28).

Epithelial cells, including mammary cells, display an additional layer of complexity as they are typically polarized and possess two distinct plasma membrane domains (6, 18, 20, 34, 44). The apical and basolateral plasma membrane domains have different protein and lipid compositions which reflect the different function of these domains. This plasma membrane polarity is established and maintained by protein sorting and specific vesicle trafficking routes in the biosynthetic and endocytic pathways. In agreement with the SNARE hypothesis, the apical and basolateral plasma membrane domains of epithelial cells contain distinct t-SNAREs (42). Two protein families have been identified as t-SNAREs, the syntaxin and SNAP-25 families. In MDCK cells, syntaxins 3 and 4 are localized at the apical or basolateral plasma membrane, respectively (21). Syntaxin 3 functions in transport from the trans-Golgi network as well as the endosomal recycling pathway, both leading to the apical plasma membrane (22). Syntaxin 2 is localized to both domains of MDCK cells (21), as is SNAP-23 (22), a ubiquitously expressed member of the SNAP-25 family (30). SNAP-23 binds to syntaxins 3 and 4 in vivo (10, 36) and is involved in biosynthetic and endocytic recycling and transcytotic pathways to both plasma membrane domains in MDCK cells (19, 23). The subcellular localization of these SNAREs is generally very similar in other epithelial cell lines and tissues although variations have been reported (5, 8-10, 32, 42).

Temporary or permanent loss of cell polarity is a common phenomenon during the development of epithelial tissues (1, 35) as well as in a number of pathological conditions (1, 7, 20). It is largely unknown how apical and basolateral membrane traffic pathways behave in epithelial cells that have lost or not yet acquired their cellular polarity under any of the above

circumstances. This is a fundamental question in cell biology and in the biology of epithelial cancers, such as breast cancer. For example, changes in these pathways may play an important role in the acquisition of the invasive phenotype of tumor cells, e.g. by mistargeting of cell adhesion molecules, or erroneous secretion of proteases that attack basement membrane and extracellular matrix proteins. It is well established that the malignancy of epithelial-derived tumors (carcinomas) correlates directly with the degree of de-differentiation. A hallmark of de-differentiation or anaplasia is the loss of cellular polarity. A better knowledge of the changes in membrane traffic pathways that occur when epithelial cells lose or gain cell polarity will help us understand normal epithelial function as well as pathologic conditions.

In the present grant we have investigated the subcellular localization of plasma membrane t-SNAREs, as part of the machinery accomplishing membrane traffic, in polarized vs. non-polarized cells.

We studied localization of SNAREs in several lines of epithelial cells, where we could manipulate their state of polarity. For mammary cells, we used the 31EG4 cell line, described below. For comparison, we used Madin Darby canine kidney (MDCK) cells, as trafficking and SNARE localization have been very well described in these cells. Basically, we have found almost identical results for both cell types. The description of results below focuses primarily on MDCK cells.

I am using a spectrum of to perturb polarity to various degrees and examine the effects on traffic. For glucocorticoids, I use dexamethasone (dex; 1 μM), which is expected to increase polarity. Addition of TGFα (l0 ng/ml) causes a loss of TER in Con8 cells, but I will investigate this in 31EG4 cells. Given the antagonist effects of TGFα and glucocorticoids, I am trying different combinations including: no dex, or dex for 48 hours, then the addition of TGFα either basolaterally or apically. TGFα and *c-myc* are synergistic, so that experiments will be designed first with the over-expression of *c-myc* alone, then TGFα added to either the apical or basolateral media. The presence of c-myc is not reported to decrease TER, therefore it will interesting to observe how they differ in their response as compared to dexamethasone. Glucocorticoids are growth inhibitory, while *c-myc* is growth promoting. *C-myc* will be expressed from the tetracycline responsive transactivator system developed by Bujard and colleagues. The original version of this system involves sequentially transfecting the cells with two plasmids, encoding first the tetracycline sensitive transactivator, and then the gene of interest (e.g. *c-myc*) under the control of a promoter that is responsive to this transactivator. Cells are grown in the presence of tetracycline, which represses the expression of the gene of interest. When tetracycline is removed, the gene of interest is turned on. K. Mostov's group has successfully used this system in MDCK cells to obtain very tightly regulated and highly inducible expression of a variety of genes.

We. observed that the previously characterized plasma membrane t-SNAREs in MDCK and mammary cells undergo similar dramatic changes in subcellular localization depending

on the degree of cellular polarity. Cells were plated at high density onto polycarbonate filters and the localization of syntaxins 2, 3, 4, and SNAP-23, as well as the tight junction protein ZO-1, were monitored at different times after plating. After 2 hours, the cells are irregularly shaped and start to form cell-cell contacts. At this stage, all plasma membrane t-SNAREs are found predominantly in intracellular vesicles in addition to a variable amount of plasma membrane staining. In approximately 10% of the cells, large intracellular vacuolar structures can be observed. After one day, the monolayer is confluent and uninterrupted circumferential tight junctions are established. A substantial portion of all SNAREs has re-localized to the plasma membrane in a polarized manner. Syntaxins 2 as well as SNAP-23 are found at both the basolateral and apical plasma membrane in addition to some remaining intracellular labeling. Syntaxin 3 is absent from the basolateral domain but localizes to the apical domain in addition to intracellular lysosomes as established previously (5, 21). Syntaxin 4, in turn, is absent from the apical domain but has partially re-localized to the basolateral domain. During the course of the experiment, until day 7, the cells grow somewhat in height and form a straight apical surface. All of the SNAREs continue to move to their final destination at their specific plasma membrane domains, however, even after 7 days some intracellular staining remains in each case as observed previously (21, 22).

This change in subcellular localization of plasma membrane t-SNAREs suggests that membrane traffic pathways leading to the plasma membrane are fundamentally altered in epithelial cells during the course of the establishment of cellular polarity.

The formation of a polarized epithelial layer can be prevented experimentally by the inhibition of E-cadherin-mediated interactions between neighboring cells (1, 2, 13). Inhibition of calcium-dependent homotypic E-cadherin binding by withdrawal of high calcium concentrations in the medium keeps MDCK cells in a non-polarized state. It has been observed previously that, when grown in low-calcium medium, MDCK cells form large intracellular vacuoles that bear ultrastructural resemblance to the apical plasma membrane including the presence of microvilli and an associated actin cytoskeleton. This compartment was termed 'vacuolar apical compartment' or VAC (38). Similar vacuoles are found in a variety of carcinomas (16, 31, 40).

We studied the subcellular localization of plasma membrane SNAREs in MDCK cells grown under these conditions. We also used the mammary cells grown under conditions where they lose polarity, as described above. A high percentage (>50%) of the cells display one or more large vacuolar compartments that are positive for the endogenous apical marker protein gp135 and are indistinguishable in appearance from previously published VACs (. Plasma membrane t-SNAREs that normally localize to the apical domain (syntaxins 2, 3, SNAP-23), co-localize with gp135 in these VACs. In contrast, the normally exclusively basolateral syntaxin 4 is excluded from gp135-positive VACs. Instead, in addition to small vesicles, syntaxin 4 is found in larger structures that resemble VACs but exclude gp135. The SNAREs that are normally localized to both apical and

basolateral plasma membrane domains (syntaxins 2, 11, SNAP-23), can be found in large gp135-negative structures (arrows) in addition to gp135-positive VACs.

Non-polarized MDCK and mammary cells stained with antibodies against syntaxin 4 and an endogenous 58 kDa basolateral plasma membrane protein (6.23.3). In addition to some plasma membrane-staining, both proteins are localized in large intracellular compartments that overlap significantly with each other (indicating that the syntaxin 4-positive compartment has a protein composition similar to the basolateral plasma membrane of polarized cells.

Together, the data suggests that although 'apical' and 'basolateral' t-SNAREs are localized intracellularly in non-polarized epithelial cells, they are nevertheless sorted to distinct compartments. These intracellular compartments resemble the respective plasma membrane domains due to the presence of 'apical' or 'basolateral' t-SNAREs as well as other plasma membrane marker proteins and an actin-based cytoskeleton.

The presence of normally apical and basolateral plasma membrane t-SNAREs in cognate intracellular compartments in non-polarized cells suggests that these SNAREs function in the membrane fusion of vesicles from incoming transport pathways that are equivalent to the plasma membrane-directed transport pathways in polarized cells. We investigated whether several proteins whose trafficking in polarized MDCK cells is well characterized are targeted to the syntaxin 3-positive VAC in non-polarized cells.

Together, these results strongly suggest that the intracellular VAC in non-polarized MDCK cells is a true cognate compartment to the apical plasma membrane in polarized cells as both contain an identical set of t-SNAREs and both receive membrane traffic from equivalent biosynthetic and endocytic pathways.

In polarized epithelial cells t-SNAREs distribute to intracellular compartments when cell polarity is lost or not yet established. To date the only functional information on the involvement of syntaxin homologues in plasma membrane traffic in polarized cells is available for syntaxin 3 which plays a role in transport from the TGN and from apical endosomes to the apical plasma membrane (17, 23). The exclusively basolateral localization of syntaxin 4 suggests that it is involved in polarized pathways to this domain. Therefore, this surprising result strongly suggests that membrane trafficking pathways that are normally directed to the plasma membrane in polarized epithelial cells undergo a fundamental shift towards intracellular compartments upon loss of cell polarity. This may have profound implications for our understanding of the pathogenesis of diseases involving a loss of epithelial polarity, e.g. the mistargeting of basement membrane proteins, proteases, integrins etc. that play a role in the pathogenesis of invasive and metastatic carcinomas (1), or the mistargeting of ion transporters, growth hormone receptors etc. in non-cancerous epithelial diseases such as polycystic kidney disease (25, 37). Also, during tubule formation - e.g. in kidney development - epithelial cells temporarily loose their cellular polarity while cell-rearrangements occur (29).

The presence of plasma membrane t-SNAREs on intracellular vacuoles in non-polarized cells indicates that plasma membrane proteins and secretory proteins are targeted into these vacuoles. Interestingly, we identified two distinct classes of vacuoles that can perhaps best be characterized by the presence of the apical t-SNARE syntaxin 3 or the basolateral t-SNARE syntaxin 4. Both compartments possess an actin cytoskeleton as shown by phalloidin staining. This distinguishes them from endosomes or other organelles which typically do not possess a prominent actin cytoskeleton but which is typical for the plasma membrane. Moreover, these compartments receive membrane traffic from cognate apical or basolateral pathways, respectively. This suggests that apical/basolateral sorting is preserved in non-polarized epithelial cells and leads to specific intracellular organelles. It has been found previously that non-polarized, fibroblastic cells also have the capability to sort apical and basolateral plasma membrane proteins (presumably in the TGN) and transport them on separate routes to the identical plasma membrane (26, 45). A major difference between non-polarized cells of epithelial and non-epithelial origin may therefore be that in the former plasma membrane and secretory proteins are retained inside the cell rather than delivered to the surface.

The vacuolar apical compartment has been described and characterized previously in non-polarized MDCK cells and other epithelial cell lines as well as in a variety of carcinomas (3, 11, 38-40). In contrast, to our knowledge, the basolateral compartment that we identified here is a novel organelle that has not been described previously, perhaps because of the lack of availability of a marker protein such as syntaxin 4. What can be the possible function of "intracellular apical and basolateral plasma membranes"?

These compartments are observed in epithelial cells that have lost their cellular polarity temporarily (e.g. sparsely seeded cells that have not yet established cell contacts) or permanently (e.g. when cell contacts are inhibited or in tumor cells). It is likely that many plasma membrane or secreted proteins are still synthesized under these conditions. Re-directing these proteins (or lipids) to intracellular compartments will prevent their secretion or their display at the cell surface. This may be a protective mechanism as it would be undesirable or even harmful to the organism to have certain proteins secreted into the interstitial space by individual epithelial cells that have lost their cell contacts. A drastic hypothetical example may be accidental secretion of hydrolytic enzymes by non-polarized pancreatic acinar cells. Indeed, a variety of hydrolytic enzymes that are normally expressed at the apical plasma membrane of Caco-2 cells were found in VACs after microtubule disruption (11). The finding that neither the apical nor basolateral vacuoles appear to be degradative, lysosomal compartments indicates that proteins may be stored in them for later use once cell contacts have been re-established. This is supported by the observation that VACs can be rapidly exocytosed as a whole from MDCK cells after re-establishment of cell-cell contacts (39) or after raising the intracellular cAMP concentration (3). A basolateral equivalent of the VAC may be used to temporarily store proteins such as integrins. A major function of many epithelia is

the directed transport of ions and solutes which is made possible by the proper apical or basolateral localization of transporters and channels. The intracellular storage of these channels and pumps may prevent accidental cell death due to excessive ion depletion or accumulation after loss of cell polarity. Interestingly, the CFTR chloride channel, while apical in polarized HT-29 cells, localizes to an intracellular compartment in non-polarized HT-29 cells (24).

In conclusion, we have shown that upon loss of cell polarity epithelial cells re-localize plasma membrane t-SNAREs and re-direct membrane trafficking pathways to intracellular cognate apical and basolateral compartments. This is likely to be a general phenomenon in epithelia and may play a fundamental role in the pathogenesis of epithelial diseases that involve a break-down of cell polarity.

7. Key Research Accomplishments.

* Localization of SNARE proteins changes as the cells change the polarization.
* Apical syntaxins localize to a VAC compartment in depolarized cells.
* Basolateral syntaxins localize to a novel intracellular compartment.

8. Reportable outcomes.

None so far.

9. Conclusions.

A basic property of mammary cells and other epithelial cells is that they are highly polarized. During oncogenesis polarity is lost, and in fact a hallmark of anaplasia is loss of polarity. Polarity requires that the machinery for transporting proteins to the correct apical or basolateral surface works correctly. We have begun to show how this is deranged in mammary carcinogenesis.

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11. Appendices.

None.